PREVALENCE OF *Salmonella* sp. IN DOMESTIC CATS IN AN ANIMAL SHELTER AND THE COMPARISON OF CULTURE AND POLYMERASE CHAIN REACTION TECHNIQUES AS DIAGNOSTIC TOOLS

A Thesis

by

MELINDA J. LEE

Submitted to the Office of Graduate Studies
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2003

Major Subject: Veterinary Microbiology

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Approved as to style and content by:

R. Bruce Simpson (Chair of Committee)	Gerald R. Bratton (Member)
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ABSTRACT

Prevalence of *Salmonella* sp. in Domestic Cats in an Animal Shelter and the Comparison of Culture and Polymerase Chain Reaction Techniques as Diagnostic Tools. (August 2003)

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Chair of Advisory Committee: Dr. R. Bruce Simpson

Previous studies on the prevalence of *Salmonella* in cats have used a variety of culture methods producing a variety of results, but none have been compared to PCR. Using a double enrichment protocol developed at the Texas Veterinary Medical Diagnostic Laboratory, the prevalence of *Salmonella* in shelter cat feces was determined in this current study. The culture protocol used included Xylose Lysine Tergitol 4 (XLT4) and MacConkey (MAC) agars with a primary enrichment in Tetrathionate broth (TTH) with iodine and a secondary enrichment in Rappoport-Vassilaidis R10 broth (RV). This study further modified an equine PCR technique and demonstrated its successful use in cats. When comparing the results of the two protocols, PCR and culture, it was found that the procedures are equally adequate at detecting the presence of *Salmonella* in cat feces. This study further confirmed that *Salmonella* is a potential hazard for families who adopt shelter cats.

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Also I need to thank Sonia Lingsweiler for developing the protocol that was used in this project. She needs to be recognized for all her hard work in its enhancement and implementation at Texas Veterinary Medical Diagnostic Lab and Texas A&M University Medical Teaching Clinical Microbiology Lab.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Salmonellosis resulting from food and water contamination is an important concern of consumers, farmers, and veterinarians. Another source of contamination not often considered is the family pet, especially cats. Cats that enter an animal shelter must be considered potential family pets. Cats can be carriers of *Salmonella* organisms clinically or subclinically, and therefore are sources of unknown infection (12). Awareness of the prevalence of *Salmonella* in shelter cats is important to human and animal health for this reason. The diagnosis of these infections is also a point of concern. Microbiological culture and DNA amplification with identification are both methods used to diagnose the presence of *Salmonella* organisms in other animal species. The accuracy and efficiency of each technique is being researched, and new types of polymerase chain reaction (PCR) procedures are being developed (5, 6, 16, 22, 26). However, to date PCR technology has not been utilized to identify *Salmonella* in cats, and culture technique has given varying results (7, 11, 23, 24).

The latest prevalence study of zoonotic organisms, including *Salmonella* infections, in cats was conducted in north-central Colorado and included a total of 206 domestic cats. Seventy-seven of the cats were from the local animal shelter (11). Samples were taken from client-owned cats and shelter animals. The statistics were divided based on cats with diarrhea and those without diarrhea within each group. Samples were collected throughout the year to determine any seasonal trends. This study

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concluded that the incidence of *Salmonella* in shelter cats was 1.3% by conventional culture techniques.

Another zoonotic prevalence study was conducted in New York State (23). Over a four month period fecal samples were collected from three shelters in the area. Only cats more than 1 month old and less than 1 year were included in the study. A total of 149 samples were collected from the shelter. Only one of the cats was positive for S*almonella* by culture assay, giving 0.7% prevalence.

From 1981-1986, 6 of the 1,100 specimens from cats submitted to the Colorado State University Diagnostic Laboratory were positive for *Salmonella*. However, this study was more directed at describing the clinical aspects of salmonellosis than prevalence (7). A 1977 paper by J. F. Timoney describes a *Salmonella* outbreak in a veterinary clinic that included 21 cats over a period of 14 weeks (25). The clinic was closed to cats after the initial 18 cases developed. A culture technique using selenite broth as an enrichment process to plate on brilliant green agar was used. The author discusses the highly transmissible nature of the organism in a hospital setting. The grooming habits of cats allow their coat to be a source of infection passed by animal care technicians. Also the oral shedding of the infected cats allowed feeding dishes to become a source of additional contamination (25).

Cats are considered to have a high immunity to *Salmonella* infections under normal circumstances (24, 25). Recently an outbreak of *Salmonella* infections occurred after a high-titer modified-live Panleukopenia virus vaccine was administered to kittens in one particular cattery (9). While it was believed the vaccination facilitated the development of salmonellosis in these kittens, the source of infection was not determined.

Again an enrichment process in selenite broth was implemented and selective media used to isolate the organisms.

Unfortunately, most studies did not indicate the exact protocol used for culture. Some denoted the use of enrichment broth and selective media but did not specify the actual procedure used. Selective media and enrichments used included selenite broth, Tetrathionate broth, Xylose Lysine Tergitol 4, and Brilliant Green agar.

Diagnosis of *Salmonella* sp. in small animals has been primarily by culturing on selective media. Recently PCR has been implemented to amplify DNA and identify organisms in large animals but not cats. A study conducted in 2000 utilized a rapid PCR assay to detect *Salmonella* organisms in equine feces (1). DNA was extracted from the feces using a spin column technique that also decreased the amount of inhibitory substances, such as bilirubin. Part of the *omp*C gene, a major structural protein of the outer membrane of *Salmonella*, was amplified and identified using southern blot hybridization and DNA sequencing. Of the 96 samples taken, two samples were positive by culture whereas 44 were positive by PCR. With PCR the prevalence of *Salmonella* in horses was between 38.5-40.6 %, depending on sample preparation. Culturing revealed only 2% prevalence in the same 96 samples. These studies strongly suggested that culture techniques may be missing positive animals in other species as well.

Another study, conducted at Texas A&M University Veterinary Medical Teaching Hospital in 1995 concluded that PCR was superior at detecting clinical and subclinical *Salmonella* infections in equines (5). Over a 17-month period environmental and fecal samples were collected. A total of 747 samples were used in the study (313 were environmental samples the other 434 fecal samples were from both inpatient horses

and samples sent to the in-house diagnostic lab). Using microbiological culture and PCR the samples were tested for the presence of *Salmonella* organisms. Of the fecal samples collected 64.5% of the horses were positive on PCR and 10.0% by conventional culture. Seasonal changes did not significantly increase or decrease the number of infections. According to this study PCR is a more sensitive and rapid way of detecting *Salmonella* in equine feces than culture.

An earlier study, by the same Texas A&M group, seeded known *Salmonella* negative equine fecal samples with specific amounts of 5 isolates (6). The samples were plated on MacConkey agar (MAC) and Brilliant Green agar (BGA) and placed in a selenite broth for enrichment. The enriched broth was plated after 24 hours of incubation to another set of MAC and BGA. Suspect colonies were run through biochemical tests to identify *Salmonella*. DNA was extracted from the same fecal samples and amplified by PCR. The products were identified by gel electrophoresis and UV transillumination. The results confirmed that PCR was as effective and a more rapid means of identifying salmonellae in equine feces than culture techniques.

A report published in 2001 described the use of PCR-hybridization and cultivation of *Salmonella* in porcine fecal and water samples (8). The study concluded that PCR was as effective as selective media culture in detecting the organism. Neither process alone was able to detect all positive samples. Of the 65 positive samples only 34 were *Salmonella* positive by both cultivation and PCR-hybridization. After double enrichment, cultivation was able to detect 97% of the positive samples where PCR was able to detect 95%. In contrast with the equine studies this study suggests that either method, culture or PCR, is equally useful for the detection of *Salmonella* in swine.

Additional studies have shown that preparation of the fecal samples for PCR has an effect on the detection rate (14). In a study titled "Comparison of Three Stool-Processing Methods for Detection of Salmonella Serogroups B, C2, and D by PCR," fecal samples were processed by three means: centrifugation, immunomagnetic separation, and selective enrichment broth cultivation. Using seeded samples each of the three processes were tested with varying amounts of *Salmonella* organisms. The conclusion was that PCR was as effective, if not better, than cultivation in the detection of *Salmonella* in fecal samples, provided the samples are properly processed.

No specific information is available on the use of PCR in the detection of *Salmonella* in cats. Other articles outlined studies using PCR to detect *Salmonella* sp. in other animal feces, human feces, food samples, and water (3, 4, 8, 16). It seems likely the techniques can be adapted to cats. From the information available, there is no question that *Salmonella* is a potential problem in shelter cats that would become pets; thus exposing pet owners and other animals to *Salmonella*. A simple and effective detection method would be extremely useful. The objectives addressed in this study are (1) to expand knowledge on the prevalence of *Salmonella* sp. in shelter cat feces, (2) to adapt the PCR technology to detect *Salmonella* sp. in cat feces, and (3) to compare the accuracy and reliability of culture detection versus PCR detection for *Salmonella* in the feces.

CHAPTER II

METHODS AND MATERIALS

Fecal samples were collected from domestic cats at the Brazos Animal Shelter in Bryan, Texas over a three month period. Other studies have included 21 up to 1100 specimens; this study used 94 samples as a manageable statistical measure of prevalence and diagnostic comparison. One hundred five samples were taken, but 11 did not follow the protocol exactly and were not included in the study. The fecal material was removed from the animal's litter box by gloved hand after natural defecation, placed in a zip-lock bag and kept cool until processed. The litter boxes were a possible source of *Salmonella* contamination but were not considered in this study due to the cleaning process instituted by the shelter staff. All fecal samples were collected within fourteen hours of defecation, and no samples were taken directly from the cat. All litter boxes were cleaned twice daily; at 7:30 am and 6:00 pm. Samples were collected at 7:00 am or 5:30 pm. The samples were taken on a weekly basis until all samples were collected. The number of samples varied weekly depending on the number of animals at the shelter and the amount of defecation. Each sample underwent specialized media culture technique (see below) and polymerase chain reaction (PCR) technique modified from those procedures used on equine feces to identify *Salmonella*.

Culture technique

Sample collection and setup

Day 1

A selective media culture protocol used in the Texas A&M University Veterinary Medical Teaching Hospital, Clinical Microbiology Lab has proven effective in isolating

the *Salmonella* species. All times and temperatures are part of this protocol (2). Samples collected at the animal shelter were taken for setup. A sterile swab was used to directly plate the fecal material onto MacConkey agar (MAC) (Becton Dickinson, Sparks Maryland) and Xylose Lysine Tergitol 4 (XLT4) (Becton Dickinson, Sparks Maryland, Niaproof, Sigma, St. Louis, Missouri) agar. A 1 gram sample of fecal matter was weighed and added to 10ml of Tetrathionate broth (TTH) (Becton Dickinson, Sparks Maryland) with the addition of 0.5 ml of iodine solution (250g Potassium Iodide (EM, Gibbstown, New Jersey), 300 g Iodine crystals(EM, Gibbstown, New Jersey), and 1 L deionized water). The MAC, XLT4, and TTH were incubated at 37º C for 24 hours for all samples collected.

Day 2

After 24 hours 1-1.5 ml of undisturbed TTH supernate was removed and used for DNA extraction (See DNA extraction section). The solid fecal matter settled to the bottom of the tube allowing the supernate to be removed with little fecal material present. After this portion of the supernate was removed the TTH was mixed thoroughly and plated onto a secondary MAC and XLT4 plate using a sterile swab. The swab was placed in Rappoport-Vassiliadis R10 broth (RV) (Becton Dickinson, Sparks Maryland). The primary XLT4 and MAC plates were visually examined for suspect *Salmonella* sp. colonies. On MAC, suspect colonies appeared as lactose negative (tan to brown) round smooth colonies. On XLT4 the suspect colonies were round smooth red colonies with black centers (13). See 'Suspect colonies' section for further diagnostic work up. The primary and secondary MAC and XLT4 plates and RV broth were incubated at 37º C for 24 hours.

Day 3

After the second 24 hour incubation, the RV was plated on tertiary MAC and XLT4 plates from the RV broth using a new sterile swab. All previous plates were examined by sight for characteristics of suspect colonies as described above and followed further diagnostic work up if present (See Suspect colonies section). The tertiary plates in addition to all other plates were incubated at 37º C for 24 hours. The RV and TTH were discarded at this time.

Day 4

All plates were examined for suspect colonies as above. Primary plates were discarded if no suspects were found. Secondary and tertiary plates were reincubated at 37º C for an additional 24 hours.

Day 5

Secondary and tertiary plates were examined for suspect colonies as described above. Secondary plates were discarded if no colonies were present. Tertiary plates were incubated an additional 24 hours at 37º C.

Day 6

Tertiary plates were viewed a final time and discarded if no suspect colonies were found. If no suspect colonies were present on any of the primary, secondary or tertiary MAC and TTH plates the sample was considered culture negative for *Salmonella* sp.

Suspect colonies

All suspect colonies, either lactose negative colonies on MAC or red colonies with black centers on XLT4, were used to inoculate Triple Sugar Iron slants (TSI) (Becton Dickinson, Sparks Maryland) and Lysine Iron Agar slants (LIA) (Becton

Dickinson, Sparks Maryland) to view biochemical reactions unique to the *Salmonella* organism. TSI and LIA were prepared by the Pathobiology Media Kitchen at Texas A&M University. The inoculated TSI and LIA were incubated at 37º C for 24 hours. If the TSI had an alkaline slant over an acidic butt with the presence of hydrogen sulfide (H2S) with or without gas and the LIA was decarboxylase positive, further biochemical tests were utilized. From the TSI slant tryptophan broth, Christensen's urea agar, and Motility S with triphenyl tetrazolium chloride medium were inoculated. These biochemical tests were incubated at 37º C for 24 hours. After incubation, all suspects that were negative for urease and indole production and were positive for motility were considered positive *Salmonella* sp. All biochemically positive *Salmonella* sp. were serogrouped using a macroscopic agglutination technique to define their "O" antigens and then sent for serotyping at the National Veterinary Service Laboratories (NVSL) in Ames, Iowa.

Polymerase Chain reaction Technique

DNA extraction

The 1-1.5 ml supernate of TTH broth that was incubated for 24 hours was centrifuged for 20 minutes at 7000 RPM. The supernate was discarded and the bacterial pellet was treated using the prescribed protocol in the DNeasy Tissue Kit (Qiagen, Valencia, California) to extract the DNA. The extracted DNA was held at -70º C until all samples were collected.

DNA amplification

The 94 samples were divided into 7 different PCR series. Each series contained 15 DNA samples in successive order, a culture positive internal control (sample number 9), a negative control (*Escherichia* coli ATCC 25922), a positive control (*Salmonella* Typhimurium ATCC 14028), and a water blank. The water blank was prepared using 25 µl of JumpStart™ Readymix™ REDTaq™ DNA polymerase (TAQ) (Sigma, St. Louis, Missouri), 1 µl of both upper (5' ATG TTG TCC TGC CCC TGG TAA GAG A 3') and lower (5' ACT GGC GTT ATC CCT TTC TCT GGT G 3') genus-specific primer (Integrated DNA Technologies, Coralville, Iowa), and 23 µl of PCR water (Sigma). (6) A master mix was prepared containing 25 µl of TAQ, 21 µl of PCR water and 1 µl of each upper and lower primer. Forty-eight microliters, per sample, of the master mix was added to the 19 labeled 2 ml PCR tubes. Two microliters of extracted DNA was added to the appropriate labeled PCR tube. The mixture was gently agitated (4). All PCR tubes were placed in a thermocycler and brought to 95º C for 3 minutes. At which time the temperature dropped to 94º C for 30 seconds, then lowered to 60º C for 30 seconds then raised to 72ºC for 45 seconds. This cycle was repeated 30 times. A final extension period lasted 10 minutes at 72º C. The products were identified by gel electrophoresis and compared to the DNA ladder, water blank, and positive and negative controls for accuracy and purity. This process is a modification of Cohen's PCR study using equine feces. Cohen's 1994 and 1996 study used 20 and 35 cycles, respectively, and a time of 35 seconds, but used the same temperatures (5, 6) Commercially developed products were used in place of individual chemicals as used in Cohen's project. This genus-

specific primer produced a band at 496 base pairs from the histidine transport operon gene (5).

Gel electrophoresis

Electrophoresis gel matrix was prepared using 1.4g agarose (Bio-Rad, Hercules, California), 99.6 ml of 1X Tris/Acetic Acid/EDTA (TAE) buffer (Bio-Rad, Hercules, California) and 3 µl ethidium bromide (Bio-Rad, Hercules, California) producing a 1.4% agar gel. The gel was then placed in an electrophoresis unit and covered with 1X TAE buffer. The first 15 wells were loaded with 10 µl of the PCR products of the samples. Well 16, 17, and 18 contained 10 µl of the reaction product from the culture positive (cp) sample, the negative control (nc), and the positive control (pc), respectively. Well 19 was filled with 5 µl of the DNA ladder (l) (Bio-Rad, Hercules, California) and well 20 was filled with 10 µl of the water blank (wb). Eighty-five volts of electricity were passed through the gel for approximately 75 minutes. After completion the gel was exposed to UV transillumination and photographed. Lanes showing a distinct band at 496 bp were considered positive for *Salmonella*. (See Appendix A for layout of gel and examples of positive samples.)

DNA sequencing

Both samples that were PCR positive but culture negative (sample numbers 65 and 95) were sent for DNA sequencing. The PCR reaction product was processed using a commercially available clean up kit (Qiagen, Valencia, California) and submitted to Texas A&M University Gene Technology Laboratory. The sequence was entered into a blast search engine and the findings were recorded in the results section.

Statistical analysis

The raw data collected from this study was analyzed by using a sign test within a commercially available add on software program used with Microsoft Excel®, to determine the statistical significance of the information obtained. Prevalence within each process (culture and PCR) was calculated separately and then compared to the other for significance. Level of significance was <0.05, therefore, a confidence interval of 95%.

CHAPTER III

RESULTS

The average age of the cats used in the study was approximately 6.5 months with a range of 4 weeks to 3 years. Each sample was from a different cat, therefore, intermittent shedding was not evaluated. All 94 fecal samples were tested using both culture and PCR techniques. Of the 94 samples, 7 were positive for *Salmonella* using both culture and PCR techniques and 84 were negative using both techniques. (Table 1)

A total of 8 samples tested positive for *Salmonella* by culture. The average age of these cats was 14 weeks (3.5 months). Six of the culture positives came from single cats; the other 2 samples came from cages with 3 cats present. When multiple cats were present in a positive cage the individual cat or cats shedding the organism could not be identified. Of the eight positive fecal samples 5 were identified from the tertiary plate and three from the secondary plate. All 8 samples were sent to the National Veterinary Service Laboratory in Ames, Iowa for serotyping. Three samples were serotype Muenchen, other serotypes identified were Bredeney, Typimurium, Newport, Mississippi, and Litchfield (Table 1). Antibiograms were performed on all culture positive samples (Table 2). The prevalence of *Salmonella* in shelter cats using the culture techniques was 8.5%.

Nine samples tested positive for *Salmonella* using the PCR technique. (Table 1) The average age of these cats was 16 weeks (4months). Seven of these samples came from cages with single cats present and 2 came from cages with 3 cats present. The prevalence of *Salmonella* in shelter cats using the PCR technique was 9.6%. There was a difference of 1.1 % prevalence when using the two different techniques. This value was not

TABLE 1. Raw data of culture and PCR results from samples taken at the animal shelter

Sample #	Age (months)	Multi/Single	Culture	PCR	Serotype
001	12	S	-	-	
002	12	S	-	-	
003	2	M	-	-	
004	2.25	M	-	-	
005	12	S	-	-	
006	2	M	-	-	
007	2	S	-	-	
008	12	M	-	-	
009	1	S	+	+	Bredeney
010	1.5	M	-	-	
011	6	S	-	-	
012	8	M	-	-	
013	2	M	-	-	
014	3	M	-	-	
015	3	M	-	-	
016	6	S	-	-	
017	12	S	-	-	
018	2.5	M	-	-	
019	5	M	-	-	
020	3	M	-	-	
021	8	M	-	-	
022	2	M	-	-	
034	3	S	-	-	
035	2	S	-	-	
036	2.5	M	-	-	
037	0.75	M	-	-	
038	24	S	-	-	
039	1.25	S	-	-	
040	2	S	-	-	
041	3	M	-	-	
042	2	M	+	+	Typhimurium
043	1.5	S	+	+	Newport
044	2	M	-	-	
045	6	S	-	-	
046	2	S	+	-	Muenchen
047	2.5	M	-	-	
048	2	M	-	-	
049	24	S	-	-	
050	2	S	-	-	
051	7	S	-	-	
052	2.5	M	-	-	
053	7	S	-	-	
054	2	M	-	-	
055	3	S	-	-	
056	2	S	-	-	

TABLE 1. Continued

Sample #	Age (months)	Multi/Single	Culture	PCR	Serotype
057	3	M	-	-	
058	2	S	-	-	
059	6	S	-	-	
060	24	S	-	-	
061	10	S	+	+	Mississippi
062	10	S	-	-	
063	1.5	S	-	-	
064	1.5	S	-	-	
065	3	S	-	+	
066	4	S	-	-	
067	3	M	-	-	
068	6	S	-	-	
069	4.5	S	-	-	
070	1	M	-	-	
071	1	M	-	-	
072	24	S	-	-	
073	12	S	-	-	
074	2	M	-	-	
075	12	S	-	-	
076	6	M	-	-	
077	1.5	S	-	-	
078	12	S	-	-	
079	2	S	-	-	
080	1	M	-	-	
081	24	S	-	-	
082	24	S	-	-	
083	36	S	-	-	
084	3	S	+	+	Muenchen
085	18	M	-	-	
086	6	S	+	+	Muenchen
087	2.5	S	-	-	
088	12	S	-	-	
089	2.5	S	-	-	
090	5	M	-	-	
091	8	S	-	-	
092	4	M	-	-	
093	2	M	-	-	
094	12	S	-	-	
095	7	S	-	+	
096	4	S	-	-	
097	12	S	-	-	
098	6	S	-	-	
099	3	M	+	+	Litchfield
100	2.5	S	-	-	

TABLE 1. Continued

Sample #	Age (months)	Multi/Single	Culture	PCR	Serotype
102	12	S	-	-	
103	12	S	-	-	
104	18	S	-	-	
105	1.75	S	-	-	
Total			8	9	
Average	6.579787234		0.085	0.096	

TABLE 2. Antibiograms for culture positive samples collected

	Sample number							
Drug	9	42	43	46	61	84	86	99
Amikacin	S	S	S	S	S	S	S	S
Amox+Clav	S	S	S	S	S	S	S	S
Ampicillin	S	S	S	S	S	S	S	S
Carbenicillin	S	S	S	S	S	S	S	S
Cephalothin	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S
Doxycycline		R	S	S	S	S	S	S
Enrofloxacin	S	S	S	S	S	S	S	S
Gentamicin	S	S	S	S				
Kanamycin	S	S	S	S	S	S	S	S
Streptomycin				S	I	S	S	I
Sulfisoxazole	R	S	R		S	S	S	S
Tetracycline	S	S	S	S	S	I	I	S
Ticarcillin+Clav	S	S	S	S	S	S	S	S
Trimethoprim+Sulfa	S	S	R	S	S	S	S	S

R- Resistant; I- Intermediate; S- Susceptible; Clav – Clavulanic acid; Sulfa- Sulfadiazine

n	94	
Mean	0.085	
95% CI	0.028	to 0.143
Variance	0.0787	
SD	0.2805	
SE	0.0289	
CV	330%	
n	94	

FIGURE 1. Descriptive summary of culture results.
CI- Confidence Interval; SD- Standard Deviation; SE- Standard Error; CV- Confidence value

n	94	
Mean	0.096	
95% CI	0.035	to 0.156
Variance	0.0875	
SD	0.2958	
SE	0.0305	
CV	309%	

FIGURE 2. Descriptive summary of PCR results.

n	94		
Difference between medians	0.000		
95.1% CI	0.000	to 0	(exact)
Sign statistic	1		
2-tailed p	1.0000	(exact)	

FIGURE 3. Sign test comparing results of both the culture technique and PCR technique.

statistically significant when comparing the two protocols using the sign test in commercially available software (www.analyse-It.com). (Fig. 1-3)

Sample number 46 was positive for *Salmonella* using the culture technique but negative when using the PCR protocol (Table 1). Samples number 65 and 95 were positive using the PCR protocol and negative by means of the culture technique. To confirm that these two samples were *Salmonella* the PCR reaction product was sent for DNA sequencing. The sequences obtained from the Texas A&M Gene Technology Laboratory are as follows for sample 65 and 95, respectively.

ANNNNNNCCCCTANNNCANNCCTTTNCGNCGNCCCACTGNCTNCATNTGNCN
CGNANGNTNCTNCCTGNCGTNCGTNCCCCTGTNAGNCACGCNCGNACGCGCT
TGCCTTTCAGNCGACGCGNACGGTCGGCTGAATATCAGNAGTTCTTCGCCAC
CACCAGACGGGAATCAGCGGCGTAAAGCTTGTCGGTAAACGCGATTTCCTGC
TGGCGCTTTTCAGTGATGGACAGCGAGGACATGATGGCATCAATTTTCTTCGC
TTTTAAAGACGGAATCAGCGCATCCAGCGGGTTTTCCACGAACGTACACTGT
GTGTTGATACGTTTGCACAGTTCTTTGGCCAGATCGATATCAAAGCCGACCAA
TTCACCTTGTGCATTTTTGGATTCAAACGGTGCGTATGTAGGATCGGTACCGA
TGCGAATCTTTTGTGGAATAGCGGCAAATGCTGCGGTAGCGCTGGAAAATGC
CAGCACCAGAGAAAGGGATAACGCCAGTAN

ANNNNNCCCCCCTNCNCANNCCTTTNCGGNCGNCCCACTGNCTNCATNTGNC
CGNAAGTNCTNCCTGCGTNCGTNCCCCTGTAGNCACGCCGNACGCGCTTGCC
TTTCAGCGACGCGACGGTCGGCTGAATATCAGNAGTTCTTCGCCACCACCAG
NACGGGAATCAGCGGCGTAAAGCTTGTCGGTAAACGCGATTTCCTGCTGGCG
CTTTTCAGTGATGGACAGCGAGGACATGATGGCATCAATTTTCTTCGCTTTTA
AAGACGGAATCAGCGCATCCAGCGGGTTTTCCACGAACGTACACTGTGTGTT
GATACGTTTGCACAGCTCTTTGGCCAGATCGATATCAAAGCCGACCAATTCAC
CTTGTGCATTTTTGGATTCAAACGGTGCGTATGTAGGATCGGTACCGATGCGA
ATCTTTTGTGGAATAGCGGCAAATGCTGCGGTAGCGCTGGAAAATGCCAGCA
CCAGAGAAAGGGATAACGCCAGTAN

A blast search of the DNA sequence concluded that sample 65 and 95 were in significant alignment with *Salmonella* Typhimurium (www.ncbi.nlm.nih.gov.com).

CHAPTER IV

SUMMARY

According to the literature, current prevalence of *Salmonella* isolated in feces from shelter cats ranges from 0.7% to 18% when using conventional culture techniques (7, 11, 23). A variety of agars and media were used in the isolation process. The conventional culture technique varied from study to study. Based on recent improvements in culture techniques, these reported values may not be accurate. The process of double enrichment and the use of improved selective agar have increased the likelihood of isolating *Salmonella* from fecal material when present (11, 23). The prevalence of *Salmonella* in cats has not been verified by polymerase chain reaction (PCR) in the investigations described in the literature. However, culturing and PCR have been compared using equine feces (1, 5, 6). These equine studies have identified PCR as a superior method of testing, based on larger numbers of positives compared to culture (1, 5). A modified PCR protocol used in the equine feces studies was successfully adapted to be used on cat feces in this study.

In this study the prevalence rate for *Salmonella* in shelter cats using culture techniques was 8.5%. This value is considerably higher than other studies (7, 11, 23). Possible reasons for this higher value include differences in protocol and seasonal influence. Seasonal influences create stressful environments for animals. The lack of water and decreased quality and quantity of food can cause an increase in the prevalence of *Salmonella* during summer months (10). Housing in an animal shelter can be stressful enough to cause suppression of immunity allowing the cats to develop salmonellosis (24). All samples were taken between June and August of 2002, when the average daytime

high temperature in Bryan/College Station was above 30ºC. The cats were housed in air conditioned rooms, but their individual environments prior to their arrival were unknown. These environmental stresses could lead to an increased number of cats affected by *Salmonella*.

The testing protocol was most likely the major difference between prevalence values in this study compared to other studies. A double enrichment process, improved selective media, and the amount of time the plates were kept all contribute to the success in demonstrating the organism in fecal matter. XLT4 is a highly selective medium that contains an anionic surfactant tergitol 4 and H_2S indicators sodium thiosulfate and ferric ammonium citrate (18). This media inhibits the growth of gram positive bacteria and fungi and partially inhibits the growth of some gram negative bacteria. *Salmonella* form distinctive red colonies with black centers, which make colony identification easier (17).

MacConkey agar is a selective medium that inhibits the growth of gram positive bacteria, by the use of bile salts and crystal violet. Lactose fermenting colonies are pink to red and non-lactose fermenting colonies are colorless or pale yellow. These easily identified colony types were tested biochemically to confirm *Salmonella* colonies. MacConkey agar allowed the growth of *Salmonella, Shigella,* and other enteric organisms, therefore, only colonies fitting the morphology were tested (13).

Double enrichment of the fecal material increases the likelihood of isolating *Salmonella* (19). Tetrathionate broth was the primary enrichment step in this protocol. The secondary enrichment was in Rappaport-Vassiliadis R10 (RV) broth. After each enrichment the broths were plated on XLT4 and MacConkey for colony isolation. Tetrathionate broth was used because it was the least likely to exclude serotypes that are

isolated in small animals and does not interfere with PCR processing (20, 21). This was an important factor since a portion of the primary enrichment was used for DNA extraction. RV broth was chosen as a secondary enrichment because it also did not exclude common serotypes which can occur with other enrichment broths (19).

Using this protocol *Salmonella* was identified within 48 hours, and a negative culture was confirmed within six days. The majority of the suspect colonies were obtained within 72 hours, and biochemically confirmed within an additional 24 hours. Five of the eight positive samples were identified and tested only from the tertiary plate, leading to the conclusion that the second enrichment was necessary to the isolation of *Salmonella* in feces.

Review of the literature does not identify a prevalence of *Salmonella* in cats based on a PCR protocol. Different primers and different methods of DNA extraction with and without pre-enrichment were used in non-feline studies (1, 4, 5, 6, 16, 26). One particular study at Colorado State University used three different commercial DNA extraction kits, and found that the DNeasy Tissue kit (Qiagen Valencia, California) was superior to others tested. The kits were evaluated against culture results to compare specificity and sensitivity (15). This is the commercial kit that was used in this protocol.

At the University of Melbourne in Australia, PCR results were compared using enriched and non-enriched starting materials on horse feces. Fecal samples were collected from horses and processed for bacterial culture which included an enrichment step in selenite broth and plating on MacConkey agar and xylose-lysine-desoxycholate agar (XLD). A portion of the fresh feces was used for DNA extraction directly. After incubation, a portion of the enrichment broth was also used for DNA extraction. PCR

was run on the two DNA extraction products and compared. The study concluded that a selective enrichment broth increased the sensitivity of the PCR. Salmonella was detected by PCR in 38.5% of the non-enriched samples and 40.6% of the enriched samples, where only 2% of the samples tested positive when evaluated only by culture methods (1).

An earlier study at Texas A&M University Veterinary Medical Teaching Hospital conducted by Cohen, et al (5) in horse feces concluded that PCR detected substantially more *Salmonella* positives than bacterial culture. DNA was extracted from an enrichment broth of tetrathionate not using a commercial kit. The bacterial culture was plated on MacConkey agar, Brilliant green agar, and put in a selenite broth for overnight enrichment. Sixty-four percent of the horses tested positive for *Salmonella* by PCR where as only 11% tested positive with culture techniques. Cohen et al. concluded that PCR was more sensitive at identifying *Salmonella* in equine feces (5).

As a comparison to the culture data, PCR was run on all 94 samples. In the present study 94 feline fecal samples were obtained; nine tested positive for *Salmonella* using PCR, and eight tested positive when evaluated with the culture technique. This difference is not statistically significant, and one can conclude that this culture protocol was as sensitive as PCR in the detection of *Salmonella* from cat feces in this small study. The culture and PCR protocol would need to be completed using a much larger sample size to confirm the results.

Previous published studies concluded that PCR was a more sensitive test for detecting the presence of *Salmonella* in feces (5, 6). However, these conclusions were based on less intensive bacterial culture techniques. With the use of the double enrichment and selective media protocol discussed in this study, culturing is more useful

and more practical than PCR. Also, previous studies had to address the idea of false-positives (1, 4, 5). Although *Salmonella* DNA was identified by PCR, it could not be concluded that this was from viable cells or that the PCR product was indeed *Salmonella* and not another enteric organism that is closely related to *Salmonella*. In this study the two PCR positive, culture negative samples were sent for DNA sequencing of the PCR amplicon. From the sequence it was determined that *Salmonella* DNA was present. However, this data is not useful beyond being informative since therapeutic strategies were not formulated and antimicrobial susceptibility tests cannot be performed on non-viable organisms. The ability to obtain an antibiogram and serotyping may become more important than general diagnosis of the presence of *Salmonella*. PCR was more efficient than culturing in respect to the amount of time taken to receive results. PCR can be completed in the same day if necessary. In some situations it is important to know that the organism is present and begin broad spectrum treatment in a timely manner.

In this study, the prevalence of *Salmonella* in shelter cats was 8.5% by culture and 9.6% by PCR. It was concluded that PCR is not significantly more sensitive at detecting *Salmonella* than the process of double enrichment and selective media. PCR could be performed in a timelier manner, being completed within 48 hours as opposed to culturing within six days. The equine protocol as modified for PCR was successfully used on cat feces.

REFERENCES

1. **Amavisit, P., G. Browning, D. Lightfoot, S. Church, G. Anderson, K. Whithear, and P. Markham.** 2001. Rapid PCR detection of *Salmonella* in horse fecal samples. Vet. Micro. **79**:63-74.

2. **Andrews, K.** 2001. Comparison of enrichment technique for isolation of *Salmonella* sp. from swine feces**.** M.S. Thesis. College of Veterinary Medicine, Dept. Pathobiology. Texas A&M University.

3. **Carli, K., C. Unal, V. Caner, and A. Eyigor.** 2001. Detection of Salmonellae in chicken feces by a combination of tetrathionate broth enrichment, capillary PCR, and capillary gel electrophoresis. J. Clinic. Micro. **39**:1871-1876.

4. **Chiu, C., and J. OU.** 1996. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. J. Clinic. Micro. **34**:2619-2622.

5. **Cohen, N., L. Martin, R. Simpson, D. Wallis, H. Neibergs.** 1996. Comparison of polymerase chain reaction and microbiological culture for detection of salmonellae in equine feces and environmental samples. AJVR. **57**:780-786.

6. **Cohen, N., H. Neibergs, D. Wallis, R. Simpson, E. McGruder, and B. Hargis.** 1994. Genus-specific detection of salmonellae in equine feces by use of polymerase chain reaction. AJVR. **55**:1049-1054.

7. **Dow, S., R. Jones, R. Henik, P. Husted.** 1989. Clinical features of salmonellosis in cats: Six cases (1981-1986). JAVMA. **194**:1464-1466.

8. **Feder, I., J. Nietfeld, J. Galland, T. Yeary, J. Sargeant, R. Oberst, M. Tamplin, and J. Luchansky.** 2001. Comparison of cultivation and PCR-hybridization for detection of *Salmonella* in porcine fecal and water samples. J. Clinic. Micro. **39**:2477-2484.

9. **Foley, J., U. Organd, D. Hirsch, A. Poland, N. Pedersen.** 1999. Outbreak of fatal salmonellosis in cats following the use of a high-titer modified-live panleukopenia virus vaccine. JAVMA **214**:67-70.

10. **Hart, R., D. Bradshaw, and J. Iveson.** 1985. *Salmonella* infections in a marsupial, the Quokka (*Setonix brachyurus*), in relation to seasonal changes in condition and environmental stress. App. Enviro. Micro. **49**:1276-1281.

11. **Hill, S., J. Cheney, G. Taton-Allen, J. Reif, C. Burns, M. Lappin.** 2000. Prevalence of enteric zoonotic organisms in cats. JAVMA. **216**:687-692.

12. **Kahrs, R. D. Holmes, and G. Poppensiek.** 1978. Diseases transmitted from pets to man: An evolving concern for veterinarians. Cornell Vet. **68**:442-459.

13. **Koneman, E., S. Allen, W. Janda, P. Schreckenberger, W. Winn.** 1998. *Salmonella*, p. 108-118. *In* Color Atlas and Textbook of Diagnostic Microbiology 5th ed. Lippincott, Philadelphia, USA.

14. **Kongmuang, U., J. Luk, and A. Lindberg.** 1994. Comparison of three stool-processing methods for detection of *Salmonella* serogroups B, C2, and D by PCR. J. Clinic. Micro. **32**:3072-3074.

15. **Kurowski, P., J. Traub-Dargatz, P. Morley, and C. Gentry-Weeks.** 2002. Detection of *Salmonella* spp in fecal specimens by the use of real-time polymerase chain reaction assay. AJVR **63**:1265-1268.

16. **Lin, J., and Hau-Yang Tsen.** 1999. Development and use of polymerase chain reaction for the specific detection of *Salmonella typhimurium* in stool and food samples. J. of Food Protec. **62**:1103-1110.

17. **Mallinson, E., R. Miller, C. de Rezende, K. Ferris, J. deGraft-Hanson, and S. Joseph.** 2000. Improved plating media for the detection of *Salmonella* species with the typical and atypical hydrogen sulfide production. J Vet Diagn Invest **12**:83-87.

18. **Miller, R., C. Tate, E. Mallinson, and J. Scherrer.** 1991. Xylose-Lysine-Tergitol 4: An improved selective agar medium for the isolation of *Salmonella*. Poultry Science **70**:2429-2432.

19. **O'Carroll, J., P. Davies, M. Correa, and B. Slenning.** 1999. Effects of sample storage and delayed secondary enrichment on the detection of *Salmonella* spp in swine feces. AJVR **60**:359-362.

20. **Read, S., R. Irwin, C. Poppe, and J. Harris.** 1994. A comparison of two methods for isolation of *Salmonella* from poultry litter samples. Poultry Science **73**:1617-1621.

21. **Schrank, I., M. Mores, J. Costa, A. Frazzon, R. Sconcini, A. Schrank, M. Vainstein, and S. Silva.** 2001. Influence of enrichment media and application of PCR based methods to detect *Salmonella* in poultry industry products and clinical samples. Vet. Micro **82**:45-53

22. **Sharma, V., and S. Carlson.** 2000. Simultaneous detection of *Salmonella* strains and *Escherichia coli* O157:H7 with fluorogenic PCR and single-enrichment-broth culture. App. And Enviro. Micro. **66**:5472-5476.

23. Spain, J., J. Scarlett, S. Wade, and P. McDonough. 2001. Prevalence of enteric zoonotic agents in cats less than 1 year old in central New York State. J. Vet. Inern. Med. **15**:33-38.

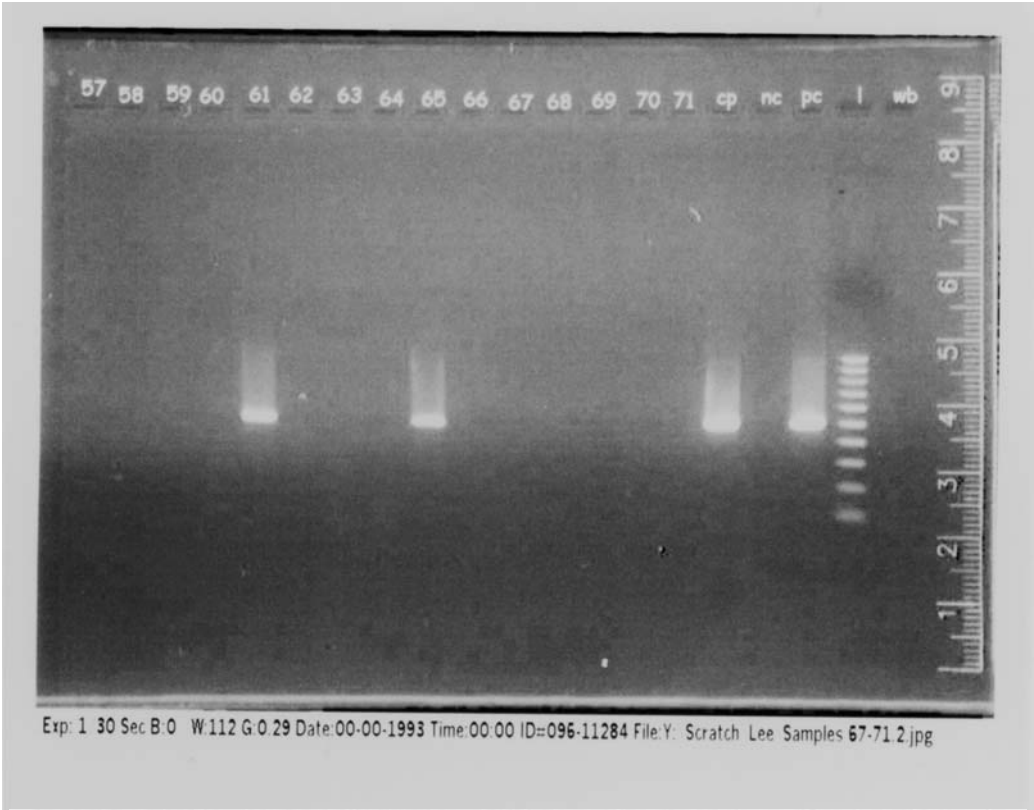
24. Timoney, J. 1976. Feline Salmonellosis. Vet. Clinics of N. Am. **6**:395-398.

25. Timoney, J., H. Neibert, and F. Scott. 1978. Feline Salmonellosis- A nosocomial outbreak and experimental studies. Cornell Vet. **68**:211-219.

26. Widjojoatmodjo, M., A. Fluit, R. Torensma, G. Verdonk, and J. Verhoef. 1992. The magnetic immuno polymerase chain reaction assay for direct detection of Salmonellae in fecal samples. J. of Clin. Micro. **30**:3195-3199.

APPENDIX A

UV TRANSILLUMINATION OF PCR AMPLICON PROCUCTS



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